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(71) Applicants *(for all designated States except US)*: **THE PENN STATE RESEARCH FOUNDATION** [US/US]; 304 Old Main, University Park, PA 16802 (US). **SUROMED, INC.** [US/US]; 2375 Garcia Avenue, Mountain View, CA 94043 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **NATAN, Michael, J.** [US/US]; 726 Casa Bonita Court, Los Altos, CA 94024 (US). **WALTON, Ian** [US/US]; 346 Lakeview Way, Redwood City, CA 94062 (US). **DIETZ, Louis, J.** [US/US]; 550 S. Shoreline Boulevard, Mountain View, CA 94041 (US). **NORTON, Scott** [US/US]; 1232 Vicente Drive, Apt.E, Sunnyvale, CA 94086 (US). **KEATING, Christine, D.** [US/US]; Box 147, Lemont, PA 16851 (US).

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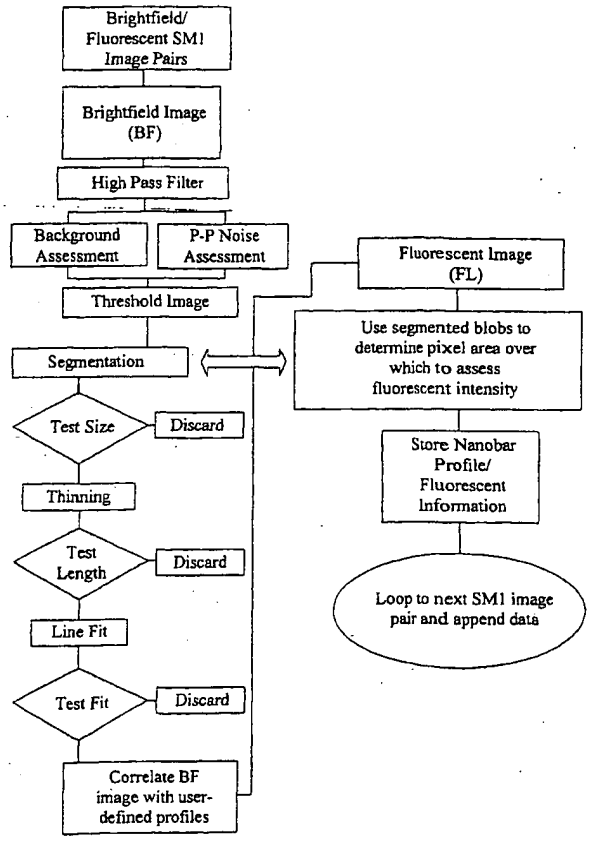
(54) Title: METHODS OF IMAGING COLLOIDAL ROD PARTICLES AS NANOBAR CODES




(57) Abstract: Methods for imaging colloidal rod particles as nanobar codes is described in which imaging or reading free-standing particles comprising a plurality of segments of length from 10 nm to 50 μm and width from 5 nm to 50 μm is performed. The segments of particles may be comprised of any material including metal, allow, a metal allow, a metal nitride, a metal chalcogenide, a metal oxide, a metal sulfide, a metal selenide, a metal telluride, polymeric materials, crystalline or non-crystalline materials.

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(74) **Agents:** **SWANSON, Barry, J.** et al.; Swanson & Bratschun, L.L.C., 1745 Shea Center Drive, Suite 330, Highlands Ranch, CO 80129 (US).

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# METHODS OF IMAGING COLLOIDAL ROD PARTICLES AS NANOBAR CODES

## FIELD OF THE INVENTION

The present invention is directed to methods of imaging nanoparticles. In certain preferred embodiments of the invention, the nanoparticles may be used to encode information and thereby serve as molecular (or cellular) tags, labels and substrates.

## BACKGROUND OF THE INVENTION

The present invention relates to methods of imaging segmented particles, assemblies of differentiable particles (which may or may not be segmented) and uses thereof.

Without a doubt, there has been a paradigm change in what is traditionally defined as bioanalytical chemistry. A major focus of these new technologies is to generate what could be called "increased per volume information content". This term encompasses several approaches, from reduction in the volume of sample required to carry out an assay, to highly parallel measurements ("multiplexing"), such as those involving immobilized molecular arrays, to incorporation of second (or third) information channels, such as in 2-D gel electrophoresis or CE-electrospray MS/MS.

Unfortunately, many of these seemingly revolutionary technologies are limited by a reliance on relatively pedestrian materials, methods, and analyses. For example, development of DNA microarrays ("gene chips") for analysis of gene expression and genotyping by Affymetrix, Incyte and similar companies has generated the wherewithal to immobilize up to 20,000 different fragments or full-length pieces of DNA in a spatially-defined 1-$cm^2$ array. At the same time, however, the use of these chips generally requires hybridization of DNA in solution to DNA immobilized on a planar surface, which is marked both by a decrease in the efficiency of hybridization (especially for cDNA) and a far greater degree of non-specific binding. It is unclear whether these problems can be completely overcome. Moreover, there is a general sense of disillusionment both about the cost of acquiring external technology and the lead-time required to develop DNA arraying internally.

A second example of how groundbreaking can be slowed by inferior tools is in pharmaceutical discovery by combinatorial chemistry. At the moment, solution phase, 5-10

μm diameter latex beads are used extensively as sites for molecular immobilization. Exploiting the widely adopted "split and pool" strategy, libraries of upwards of 100,000 compounds can be simply and rapidly generated. As a result, the bottleneck in drug discovery has shifted from synthesis to screening, and equally importantly, to compound identification, (i.e., which compound is on which bead?). Current approaches to the latter comprise "bead encoding", whereby each synthetic step applied to a bead is recorded by parallel addition of an organic "code" molecule; reading the code allows the identity of the drug lead on the bead to be identified. Unfortunately, the "code reading" protocols are far from optimal: in every strategy, the code molecule must be cleaved from the bead and separately analyzed by HPLC, mass spectrometry or other methods. In other words, there is at present no way to identify potentially interesting drug candidates by direct, rapid interrogation of the beads on which they reside, even though there are numerous screening protocols in which such a capability would be desirable.

Two alternative technologies with potential relevance both to combinatorial chemistry and genetic analysis involve "self-encoded beads", in which a spectrally identifiable bead substitutes for a spatially defined position. In the approach pioneered by Walt and co-workers, beads are chemically modified with a ratio of fluorescent dyes intended to uniquely identify the beads, which are then further modified with a unique chemistry (e.g. a different antibody or enzyme). The beads are then randomly dispersed on an etched fiber array so that one bead associates with each fiber. The identity of the bead is ascertained by its fluorescence readout, and the analyte is detected by fluorescence readout at the same fiber in a different spectral region. The seminal paper (Michael *et al.*, Anal. Chem. 70, 1242-1248 (1998)) on this topic points out that with 6 different dyes (15 combinations of pairs) and with 10 different ratios of dyes, 150 "unique optical signatures" could be generated, each representing a different bead "flavor". A very similar strategy is described by workers at Luminex, who combine flavored beads ready for chemical modification (100 commercially available) with a flow cytometry-like analysis. (See, e.g., McDade *et al.*, Med. Rev. Diag. Indust. 19, 75-82 (1997)). Once again, the particle flavor is determined by fluorescence, and once the biochemistry is put onto the bead, any

spectrally distinct fluorescence generated due to the presence of analyte can be read out. Note that as currently configured, it is necessary to use one color of laser to interrogate the particle flavor, and another, separate laser to excite the bioassay fluorophores.

A more significant concern with self-encoded latex beads is the limitations imposed by the wide bandwidth associated with molecular fluorescence. If the frequency space of molecular fluorescence is used both for encoding and for bioassay analysis, it is hard to imagine how, for example, up to 20,000 different flavors could be generated. This problem might be alleviated somewhat by the use of combinations of glass-coated quantum dots, which exhibit narrower fluorescence bandwidths. (See, e.g. Bruchez *et al.*, Science, 281, 2013-2016 (1998)). However, these "designer" nanoparticles are quite difficult to prepare, and at the moment, there exist more types of fluorophores than (published) quantum dots. If, however, it were possible to generate very large numbers of intrinsically-differentiable particles by some means, then particle-based bioanalysis would become exceptionally attractive, insofar as a single technology platform could then be considered for the multiple high-information content research areas; including combinatorial chemistry, genomics, and proteomics (via multiplexed immunoassays).

Previous work has originally taught how metal can be deposited into the pores of a metallized membrane to make an array of metal nanoparticles embedded in the host. Their focus was on the optical and/or electrochemical properties of these materials. A similar technique was used to make segmented cylindrical magnetic nanoparticles in a host membrane, where the composition of the particles was varied along the length. In no case, however, have freestanding, rod-shaped nanoparticles with variable compositions along their length been prepared. Indeed, "freestanding" rod-shaped metal nanoparticles of a single composition, in which the length is at least one micron, have never been reported. Likewise, freestanding rod-shaped metal nanoparticles not embedded or otherwise contained within such host materials have never been reported.

## SUMMARY OF THE INVENTION

Rod-shaped nanoparticles have been prepared whose composition is varied along the length of the rod. These particles are referred to as nanoparticles or nanobar codes, though in reality some or all dimensions may be in the micron size range. The present invention is directed to methods of imaging or reading such nanoparticles.

The imaging or reading of nanoparticles according to the present invention may have two components. The primary component of imaging the nanobar codes of the present invention is the ability to identify the specific type or flavor of nanobar code. The nanobar codes of the present invention are defined, in part, by the ability to be differentiated from other nanobar codes, or the ability to encode information. The first component of the imaging of the present invention is, therefore, this ability to identify the specific flavor of nanobar code.

The second component of the imaging or reading of the nanobar codes of the present invention is applicable in those embodiments where the nanobar codes are used, for example, in molecular assays. In these embodiments, the nanobar codes serve as a tag to identify the specific assay being conducted. It is necessary, therefore, to be able to read the tag (the first component above) and also to be able to detect or read the result of the assay. In these cases, the results from the imaging or reading of the first component and the reading of the assay must be correlated so that the assay type and the assay result are correctly associated with each other.

The present invention includes methods of imaging or reading free-standing particles comprising a plurality of segments, wherein the particle length is from 10 nm to 50 μm and particle width is from 5 nm to 50 μm. The segments of the particles of the present invention may be comprised of any material. Included among the possible materials are a metal, any metal chalcogenide, a metal oxide, a metal sulfide, a metal selenide, a metal telluride, a metal alloy, a metal nitride, a metal phosphide, a metal antimonide, a semiconductor, a semi-metal, any organic compound or material, any inorganic compound or material, a particulate layer of material or a composite material. The segments of the particles of the present invention may be comprised of polymeric materials, crystalline or non-crystalline materials, amorphous materials or glasses. In certain preferred embodiments of the invention, the particles are "functionalized" (e.g., have their surface coated with IgG antibody). Such functionalization may be attached on selected or all segments, on the body or one or both tips of the particle. The functionalization may actually coat segments or the entire particle. Commonly, such functionalization may include organic compounds, such as an antibody, an antibody fragment, or an oligonucleotide, inorganic compounds, and combinations thereof. Such functionalization may also be a detectable tag or comprise a species that will bind a detectable tag.

Also included within the present invention are methods of imaging or reading an assembly or collection of particles comprising a plurality of types of particles, wherein each particle is from 10 nm to 50 μm in length and is comprised of a plurality of segments, and wherein the types of particles are differentiable. In the preferred embodiments, the particle types are differentiable based on differences in the length, width or shape of the particles and/or the number, composition, length or pattern of said segments. In other embodiments, the particles are differentiable based on the nature of their functionalization or physical properties (e.g., as measured by mass spectrometry or light scattering).

The present invention includes a method for reading information that has been encoded about a material or product (e.g., paint, rubber, metal, wood, textiles, gunpowder, paper, plastics, glass, polystyrene beads, etc.) when a free standing particle that encodes the information has been incorporated within or attached to said material or product, comprising incorporating within or attaching to said material or product a free standing particle that encodes the information, said particle comprising a plurality of segments, wherein the particle length is from 10 nm to 50 μm and the particle width is from 5 nm to 50 μm; and wherein said encoded information is based on the length, width or shape of the particle and/or the number, composition, length or pattern of the segments.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is an image that shows an assembly of six types of nanobar codes. The figure diagramatically illustrates the six flavors of nanobar codes, A-F, and the image is labeled to show which of the nanobar codes in the image correspond to the various flavors or types of nanobar code.

Figure 2 shows a graph of reflectivity versus wavelength for bulk Pt and Au.

Figure 3A is an image of a collection of Ag-/Au- nanorods at 400 nm and Figure 3B is an image of the same collection at 600 nm.

Figure 4 is an image taken from an optical microscope in reflected light mode of a 9-striped bar code (Au-/Ag-/Au-/Ag-/Au-/Ag-/Au-/Ag-/Au) of the present invention.

Figure 5 demonstrates simultaneous bar code detection by reflectivity and analyte quantitation by fluorescence. Each of the images is a mixture of striped nanorods as described in Example 1. Figure 5A is imaged at the wavelength of FITC emission with a bandpass filter. Figure 5B is imaged at the wavelength of Texas Red. Figure 5C is a reflectivity image at 400 nm.

Figure 6 is a flow chart depicting alternative pathways for nanobar code image analysis.

Figure 7 is the primary flow chart of an embodiment of the nanobar code imaging analysis of the present invention.

Figure 8 is a schematic diagram of an MLSC apparatus for use with the present invention.

Figure 9 schematically depicts a flow system for imaging nanoparticles.

## DETAILED WRITTEN DESRIPTION OF THE INVENTION

The present invention is directed to methods of imaging or reading nanoparticles. Such nanoparticles and their uses are described in detail in United States Utility Application Serial No. 09/598,395, filed June 20, 2000, entitled "Colloidal Rod Particles as Nanobar Codes", incorporated herein in its entirety by reference. Filed concurrently with the present application, and also incorporated herein in their entirety by reference, are two United States Utility Applications entitled "Methods of Manufacture of Colloidal Rod Particles as Nanobar Codes" and "Colloidal Rod Particles as Nanobar Codes." The present application is filed as a Continuation-in-Part of the 09/598,395 application.

Because bar coding is so widely-used in the macroscopic world, the concept has been translated to the molecular world in a variety of figurative manifestations. Thus, there are "bar codes" based on analysis of open reading frames, bar codes based on isotopic mass variations, bar codes based on strings of chemical or physical reporter beads, bar codes based on electrophoretic patterns of restriction-enzyme cleaved mRNA, bar-coded surfaces for repeatable imaging of biological molecules using scanning probe microscopies, and chromosomal bar codes (a.k.a. chromosome painting) produced by multi-chromophore fluorescence *in situ* hybridization. All these methods comprise ways to code biological information, but none offer the range of advantages of the *bona fide* bar codes of the present invention, transformed to the nanometer scale.

The particles to be imaged or read according to the present invention are alternately referred to as nanoparticles, nanobar codes, rods and rod shaped particles. To the extent that any of these descriptions may be considered as limiting the scope of the invention, the label applied should be ignored. For example, although in certain embodiments of the invention, the particle's composition contains informational content, this is not true for all embodiments of the invention. Likewise, although nanometer-sized

particles fall within the scope of the invention, not all of the particles of the invention fall within such size range.

In preferred embodiments of the present invention, the nanobar code particles are made by electrochemical deposition in an alumina or polycarbonate template, followed by template dissolution, and typically, they are prepared by alternating electrochemical reduction of metal ions, though they may easily be prepared by other means, both with or without a template material. Typically, the nanobar codes have widths between 30 nm and 300 nanometers, though they can have widths of several microns. Likewise, while the lengths (i.e. the long dimension) of the materials are typically on the order of 1 to 15 microns, they can easily be prepared in lengths as long as 50 microns, and in lengths as short as 20 nanometers. In some embodiments, the nanobar codes comprise two or more different materials alternated along the length, although in principle as many as dozens of different materials could be used. Likewise, the segments could consist of non-metallic material, including but not limited to polymers, oxides, sulfides, semiconductors, insulators, plastics, and even thin (i.e., monolayer) films of organic or inorganic species.

When the particles of the present invention are made by electrochemical deposition the length of the segments (as well as their density and porosity) can be adjusted by controlling the amount of current passed in each electroplating step; as a result, the rod resembles a "bar code" on the nanometer scale, with each segment length (and identity) programmable in advance. Other forms of electrochemical deposition can also yield the same results. For example, deposition can be accomplished via electroless processes and by controlling the area of the electrode, the heterogeneous rate constant, the concentration of the plating material, and the potential. The same result could be achieved using another method of manufacture in which the length or other attribute of the segments can be controlled. While the diameter of the rods and the segment lengths are typically of nanometer dimensions, the overall length is such that in preferred embodiments it can be visualized directly in an optical microscope, exploiting the differential reflectivity of the metal components.

The synthesis and characterization of multiple segmented particles is described in Martin *et al.*, Adv. Materials 11:1021-25 (1999). The article is incorporated herein by reference in its entirety. Also incorporated herein by reference in their entirety are United States Provisional Application Serial No. 60/157,326, filed October 1, 1999, entitled "Self Bar-coded Colloidal Metal Nanoparticles"; United States Provisional Application Serial

No. 60/189,151, filed March 14, 2000, entitled "Nanoscale Barcodes"; United States Provisional Application Serial No. 60/190,247, filed March 17, 2000, entitled "Colloidal Rod Particles as Barcodes"; and United States Provisional Application Serial No. 60/194,616, filed April 5, 2000, entitled "Nanobarcodes: Technology Platform for Phenotyping."

The particles of some embodiments of the present invention are defined in part by their size and by the existence of at least 2 segments. The length of the particles can be from 10 nm up to 50 μm. In preferred embodiments the particle is 500 nm - 30 μm. In the most preferred embodiments, the length of the particles of this invention is 1-15 μm. The width, or diameter, of the particles of the invention is within the range of 5 nm - 50 μm. In preferred embodiments the width is 10 nm - 1 μm, and in the most preferred embodiments the width or cross-sectional dimension is 30 nm - 500 nm.

As discussed above, the particles of the present invention are characterized by the presence of at least two segments. A segment represents a region of the particle that is distinguishable, by any means, from adjacent regions of the particle. Segments of the particle bisect the length of the particle to form regions that have the same cross-section (generally) and width as the whole particle, while representing a portion of the length of the whole particle. In preferred embodiments of the invention, a segment is composed of different materials from its adjacent segments. However, not every segment needs to be distinguishable from all other segments of the particle. For example, a particle could be composed of 2 types of segments, e.g., gold and platinum, while having 10 or even 20 different segments, simply by alternating segments of gold and platinum. A particle of the present invention contains at least two segments, and as many as 50. The particles of the invention preferably have from 2-30 segments and most preferably from 3-20 segments. The particles may have from 2-10 different types of segments, preferably 2 to 5 different types of segments.

A segment of the particle of the present invention is defined by its being distinguishable from adjacent segments of the particle. The ability to distinguish between segments includes distinguishing by any physical or chemical means of interrogation, including but not limited to electromagnetic, magnetic, optical, spectrometric, spectroscopic and mechanical. In certain preferred embodiments of the invention, the method of interrogating between segments is optical (reflectivity).

Adjacent segments may even be of the same material, as long as they are distinguishable by some means. For example, different phases of the same elemental material, or enantiomers of organic polymer materials can make up adjacent segments. In addition, a rod comprised of a single material could be considered to fall within the scope of the invention if segments could be distinguished from others, for example, by functionalization on the surface, or having varying diameters. Also particles comprising organic polymer materials could have segments defined by the inclusion of dyes that would change the relative optical properties of the segments.

The composition of the particles of the present invention is best defined by describing the compositions of the segments that make up the particles. A particle may contain segments with extremely different compositions. For example, a single particle could be comprised of one segment that is a metal, and a segment that is an organic polymer material.

The segments of the present invention may be comprised of any material. In preferred embodiments of the present invention, the segments comprise a metal (e.g., silver, gold, copper, nickel, palladium, platinum, cobalt, rhodium, iridium); any metal chalcognide; a metal oxide (e.g., cupric oxide, titanium dioxide); a metal sulfide; a metal selenide; a metal telluride; a metal alloy; a metal nitride; a metal phosphide; a metal antimonide; a semiconductor; a semi-metal. A segment may also be comprised of an organic mono- or bilayer such as a molecular film. For example, monolayers of organic molecules or self assembled, controlled layers of molecules can be associated with a variety of metal surfaces.

A segment may be comprised of any organic compound or material, or inorganic compound or material or organic polymeric materials, including the large body of mono and copolymers known to those skilled in the art. Biological polymers, such as peptides, oligonucleotides and carbohydrides may also be the major components of a segment. Segments may be comprised of particulate materials, e.g., metals, metal oxide or organic particulate materials; or composite materials, e.g., metal in polyacrylamide, dye in polymeric material, porous metals. The segments of the particles of the present invention may be comprised of polymeric materials, crystalline or non-crystalline materials, amorphous materials or glasses.

Segments may be defined by notches on the surface of the particle, or by the presence of dents, divits, holes, vesicles, bubbles, pores or tunnels that may or may not

contact the surface at the particle. Segments may also be defined by a discernable change in the angle, shape or density of such physical attributes or in the contour of the surface. In embodiments of the invention where the particle is coated, for example with a polymer or glass, the segment may consist of a void between other materials.

The length of each segment may be from 10 nm to 50 μm. In preferred embodiments the length of each segment is 50 nm to 20 μm. The interface between segments, in certain embodiments, need not be perpendicular to the length of the particle or a smooth line of transition. In addition, in certain embodiments the composition of one segment may be blended into the composition of the adjacent segment. For example, between segments of gold and platinum, there may be a 5 to 50 nm region that is comprised of both gold and platinum. This type of transition is acceptable so long as the segments are distinguishable. For any given particle the segments may be of any length relative to the length of the segments of the rest of the particle.

As described above, the particles of the present invention can have any cross-sectional shape. In preferred embodiments, the particles are generally straight along the lengthwise axis. However, in certain embodiments the particles may be curved, bent, or helical. The ends of the particles of the present invention may be flat, convex or concave. In addition, the ends may be spiked, jagged, or pencil tipped. Sharp-tipped embodiments of the invention may be preferred when the particles are used in Raman spectroscopy applications or others in which energy field effects are important. The ends of any given particle may be the same or different. Similarly, the contour of the particle may be advantageously selected to contribute to the sensitivity or specificity of the assay (e.g., a undulating contour will be expected to enhance "quenching" of fluorophores located in the troughs).

In many embodiments of the invention, an assembly or collection of particles is prepared. In certain embodiments, the members of the assembly are identical, while in other embodiments, the assembly is comprised of a plurality of different types of particles. In embodiments of the invention comprising assemblies of identical particles, the length of particles for particles in the 1 μm - 15 μm range may vary up to 10%. Segments of 10 nm in length will vary ± 5 nm while segments in 1 μm range may vary up to 10%. The width of such particles may vary between 10 and 100% preferably less than 50% and most preferably less than 10% .

The present invention includes imaging and distinguishing between members of assemblies or collections of nanobar codes made up of a plurality of particles that are differentiable from each other. Assembly or collection, as used herein, does not mean that the nanoparticles that make up such an assembly or collection are ordered or organized in any particular manner. Such an assembly is considered to be made up of a plurality of different types or "flavors" of particles. In some such assemblies, each of the nanobar codes of the assembly may be functionalized in some manner. In many applications, the functionalization is different and specific to the specific flavor of nanoparticle. The assemblies of the present invention can include from 2 to $10^{10}$ different and identifiable nanoparticles. Preferred assemblies include more than 10, more than 100, more than 1,000 and, in some cases, more than 10,000 different flavors of nanoparticles. The particles that make up the assemblies or collections of the present invention are segmented in most embodiments. However, in certain embodiments of the invention the particles of an assembly of particles do not necessarily contain a plurality of segments.

In the embodiments of the present invention where the nanobar codes contain some informational content, or where an assembly of nanobar codes contain a plurality of types of particles, the types of particles are differentiable apart from the nature of the functionalization of each particle type. In this invention, the ability to differentiate particle types or to interpret the information coded within a particle is referred to as "interrogating" or "reading" or "differentiating" or "identifying" the nanoparticle. Such differentiation of particles may be read by any means, including optical means, electronic means, physical means, chemical means and magnetic means. The particle may even contain different sections that will be interrogated or read by different means. For example, one half of a particle may be comprised of segments whose pattern and shapes can be read by optical means, and the other half may be comprised of a segment whose pattern and shapes may be read by magnetic means. In another example, two or more different forms of interrogation may be applied to a particle, e.g., the shape or length of the particle may be read by optical means and the segment patterns by magnetic means. Such multiple forms of interrogation may be applied to the entire particle, a given segment of the particle, or a combination thereof.

In certain embodiments of the invention, the functional unit or functionalization of the particle comprises a detectable tag. A detectable tag is any species that can be used for detection, identification, enumeration, tracking, location, positional triangulation, and/or

quantitation. Such measurements can be accomplished based on absorption, emission, generation and/or scattering of one or more photons; absorption, emission generation and/or scattering of one or more particles; mass; charge; faradoic or non-faradoic electrochemical properties; electron affinity; proton affinity; neutron affinity; or any other physical or chemical property, including but limited to solubility, polarizability, melting point, boiling point, triple point, dipole moment, magnetic moment, size, shape, acidity, basicity, isoelectric point, diffusion coefficient, or sedimentary coefficient. Such molecular tag could be detected or identified via one or any combination of such properties.

The particles of the present invention may be used for a variety of applications. There are two major classifications of uses: those embodiments where the segments of the particle have informational content, and those where the segments do not have informational content. In those embodiments where the segments have informational content, the best analogy is to macroscopic bar coding. Conventional bar coding provides for a strip of black lines whereby the distance between lines and thickness of the lines are used to "code" a significant amount of information. Because of the small size of the particles of the present invention, in certain embodiments it is possible to use the particles of the invention as molecular tags. Unique identifying tags that can be "read" can be attached to any material including to molecular entities in order to track molecular events.

A key property of certain embodiments of the particles of the present invention is that when the nanorods are segmented, differences in the reflectivities of the component metals can be visualized by optical microscopy. Thus, for example, in a segmented Au-/Pt-/Au rod of 200 nm in diameter and 4-5 microns in overall length, the segments are easily visualized in a conventional optical microscope, with the Au segments having a gold lustre, and the Pt segments having a more whitish, bright lustre. Another key property of the materials is that the length of the segments, when they are prepared by alternating electrochemical reduction of two or more metal ions in a membrane, is controlled (and defined) completely by a) the composition of the solution and b) the number of Coulombs of charge that are passed in each step of an electrochemical reduction. The number of the segments can be varied at will. Likewise the diameter and cross-sections of the particles can be controlled by selecting (or coating) membranes with appropriate pore size and shape. **Figure 1** shows an image of a collection of nanoparticles of the present invention comprised of six different types or flavors of nanoparticles. This

image demonstrates the ability to differentiate between the different types of nanobar codes in a collection of nanobar codes.

The ability to identify nanobar codes via their reflectivity and the ability to modify their surfaces with biomolecules allows nanobar codes to be used as optical tags.

The nanobar code particles of the present invention can be used as tags in virtually any application where fluorescent tags or quantum dots are now used, or in conjunction with any assay or analytical procedure familiar to those skilled in the art. For example, a standard sandwich type immunoassay can be conducted wherein the nanobar code particle of the present invention serves as the stationary phase, or potentially even the "tag". The surface of the particle is functionalized to include an antibody to an analyte. When an analyte binds to said antibody a second fluroescently labeled antibody signals the presence of the analyte. The use of the nanobar code allows multiplexing by enabling the ability to conduct large numbers of assays at the same time. Positive signals can be identified and the nanobar code read to determine which analyte has been detected. The same general principle can be used with competitive assays as they are widely known to those skilled in the art.

Like macroscopic bar codes, which are based on difference in contrast of closely spaced lines of ink or other materials, in many embodiments the nanobar codes of the present invention are distinguished or identified based on different patterns of reflectivities of the various segments. What distinguishes nanobar codes from other types of optical tags, or indeed from any type of tag ever applied to a molecular system (including isotopic tags, radioactive tags, molecular tags for combinatorial beads, fluorescence-based tags, Raman-based tags, electrochemical tags, and other tags known to those of skill in the art,) is the essentially unlimited variability. With the ability to use 7 or more different metals, 20 or more different segments, and 4 or more different segment lengths, and with 3 or more different rod widths, there are essentially an infinite number of different nanobar codes that can be prepared. Even with just two types of metals and just 10 segments, with just one segment length, and with just one rod width, over a thousand different types (henceforth "flavors") of nanobar codes can be prepared.

The particles of the present invention can be read using existing instrumentation, e.g., chemical force microscopy, optical readers, etc. However, instrumentation and software specifically designed to identify nanobar codes are also within the scope of this invention. Specifically included within the scope of the invention are modified Micro

Volume Laser Scanning Cytometry (MLSC) apparatus and modified flow cytometer apparatus that can be used to image or read nanobar codes.

The wavelength dependence of metal reflectivity presents another interesting and powerful detection format for nanobar codes. For example, if one looks at the %reflectivity vs. wavelength plots of Au and Pt (**Figure 2**), there is a crossing point. In other words, there is a wavelength at which the reflectivities of the metals are the same. This is referred to as a reflectivity isosbestic. At the reflectivity isosbestic, the reflectivity of a nanobar code is uniform, even though there is variation in composition along its length. Importantly, this reflectivity isosbestic can be perturbed by binding particles (e.g., metal or organic) to the nanobar code surface or by other means. Thus, molecular recognition or any other events that lead to binding (or debinding) of particles to the surface of a nanobar code can be used to detect that event by reflectivity. For example, consider 100 different flavors of nanobar codes, each associated with a different capture antibody in a solution that contains the hundred corresponding secondary antibodies, each tagged with a colloidal Ag nanoparticle. The particles nanobar codes are observed at the reflectivity isosbestic, and all appear uniform. Introduction of a solution containing one or more of the antigens will lead to formation of antibody-antigen-antibody complexes at certain nanobar codes. At these and only these nanobar codes, the metals' reflectivities will be perturbed (differentially), and there will no longer be an isosbestic, meaning that those nanobar codes can be identified by their segmented patterns. Reflectivity isosbestics, and perturbations thereof, thus allow rapid screening in complex, multiplexed assays, in that a "signal" (e.g., a discernable pattern) can be expected to occur only for a small subset of the nanobar code flavor population.

It is important to note that beyond simple identification using reflectivity isosbestics, the intensity of the differential reflectivity in the aforementioned example can be used for quantitation.

**Figure 2** shows a plot of reflectivity vs. wavelength for bulk Pt and Au. Because of the finite-size effect, the plot would differ somewhat for nanoparticles, but the two relevant points are that (a) at most wavelengths, the reflectivity is different (thereby providing a contrast mechanism) and (b) at about 600 nm, they have the same reflectivity (a reflectivity isobestic). **Figure 3** shows images of gold and silver nanoparticles at 400 nm and 600 nm, demonstrating this principle. It is well-known how bulk reflectivity of the thin metal films in the visible region of the electromagnetic spectrum depends on

morphology; this is especially so for noble metal surfaces, in which nanometer-scale roughness features can act as scattering sites for surface plasmons. This leads to greatly enhanced antigen sensitivity. Translating this concept to bar codes, the idea is that molecular recognition-induced binding of colloidal Au to the Au segments of a colloidal bar code will change the bulk reflectivity, and at a reflectivity isosbestic, will lead to reflectivity contrast. One could thus consider the colloidal metal nanoparticles to be contrast agents. This reflectivity contrast mechanism -- which is essentially a solution analogue to surface plasmon resonance -- has the potential to be exquisitely sensitive. Using commercial instrumentation, it has been shown that detection of one 40-nm diameter colloidal Au particle per 10 square microns is easily attainable. Because the surface area of the bar code is much less than 1 $\mu m^2$, it is anticipated that binding of a single particle to a single segment will be detectable; furthermore, methods to limit the colloidal coverage of a biomolecule of interest to one per particle are possible.

Another very significant aspect of the detection mechanism of this embodiment is that bar codes that bind colloidal Au will exhibit contrast, an enormous benefit in screening. Thus, one could have in solution 100 different types of bar codes, each derivatized with a different capture molecule and the appropriate colloidal Au-tagged recognition elements. Interrogated at the reflective isobestic, the rods would all be featureless. Introduction of a solution with one unknown would cause just one type of bar code to light up, with the analyte identified by the code, and the analyte concentration defined by the integrated reflectivity change. Like the molecular beacon approach used to detect longer oligonucleotides in molecular biology by selective interruption of fluorescence quenching (see, e.g., Piatek *et al.*, Nature Biochem. 16, 359-363 (1998)), this method singles out particles where chemical events have occurred, with the added advantage that the method is completely general, and can be applied to the following systems (among others), oligo-oligo, antibody-antigen, and ligand-receptor systems. Although this effect has been discussed above with respect to colloidal Au, it is also observed with other metal particles which are observed to lead to a change in reflectivity as a function of various conditions (e.g., heating, pH, proximity to other species, etc.)

Thus, at least two different ways to do analyte quantitation are envisioned, one in which quantitation is made on the basis of the fluorescence intensity emanating from a particular nanobar code (which could derive from a molecular or particulate fluorescent tag), or from the intensity of the differential reflectivity. In both cases, reflectivity is also

used to identify the nanobar code. It should be noted, however, that a variety of other schemes can be used both for analyte quantitation and nanobar code flavor identification. For analyte quantitation, these could include, but are not limited to, fluorescent tags, electrochemical tags, radioactive tags, mass tags (such as those used in mass spectrometry), other molecular tags (such as those used in combinatorial chemistry), or other particulate tags. Indeed, nanobar codes appear to be compatible with all known analyte detection mechanisms. Likewise, for nanobar code identification, a variety of detection mechanisms can be used, including but not limited to optical detection mechanisms (absorbance, fluorescence, Raman, hyperRaman, Rayleigh scattering, hyperRayleigh scattering, CARS, sum frequency generation, degenerate four wave mixing, forward light scattering, back scattering, or angular light scattering), scanning probe techniques (near field scanning optical microscopy, AFM, STM, chemical force or lateral force microscopy, and other variations), electron beam techniques (TEM, SEM, FE-SEM), electrical, mechanical, and magnetic detection mechanisms (including SQUID). Although the discussion above refers to the reflectivity isobestic point, nanobar code assays can make use of other types of isobestic points to achieve the same types of advantages (e.g., conductivity isobestic). Indeed, more generally, any property that can change from material to material can be similarly exploited, provided means exist to manipulate the conditions so that the property is the same in the materials.

The sensitivity of hyperRaleigh scattering may make it a particularly useful interrogation technique for reading the nanobar codes of the present invention. For example, see, Johnson *et al.*, The Spectrum, 13, 1-8 (2000), incorporated herein by reference.

Moreover, it should be clear that while many embodiments of the invention are directed to quantitation, nanobarcoding, like its macroscopic counterpart, can be used for tracking, locating, or following matter in a non-quantitive fashion. Indeed, these particles can be used to label, detect, quantify, follow, track, locate, inventory, recognize, compare, identify, spot, make out, classify, see, categorize, label or discover matter, from sizes as small as individual molecules to as large as humans, cars, tanks, bridges, buildings, etc.

Moreover, it should be clear that many embodiments of the invention are directed to utility in biological systems, nanobar coding is of equal utility in the aforementioned ways for non-biological systems, including but not limited to chemicals, molecules, materials, particles, paints, fasteners, tires, paper, documents, pills, and so on. When used

as tag or label, the particles of the present invention can be associated in any way with the material it is labeling. The particular tag can be selected and identified so that it provides information regarding the material it is associated with. For example, a tag within a paint may encode the date of manufacture, the chemicals used in the paint mix, the name of the manufacturer, photodynamic characteristics of the paint or any number of other pieces of information. By saying that the nanobar code encodes information does not imply that you can read the information off of the particle. It, in most embodiments, will indicate a specific type of nanobar code, and reference would then be made to records concerning that type of nanobar code.

In one embodiment of the invention the nanoparticles are not comprised of segments, but are differentiable based on their size, shape or composition. In this embodiment, each particle in an assembly or collection of particles has at least one dimension that is less than 10 μm. In preferred embodiments, the particles have one dimension less than 500 nm, and more preferably less than 200 nm.

Such an assembly of particles, which can be made up of any material, is comprised of at least 2, preferably at least 3, and most preferably at least 5 types of particles, wherein each type of particle is differentiable from each other type of particle. In the preferred embodiment, since the types of particles may be comprised of a single material and since different types of particles may be comprised of the same material as other types of particles in the assembly, differentiation between the types is based on the size or shape of the particle types. For example, an assembly of particles of the present invention may be comprised of 5 different types of gold rod-shaped nanoparticles. Although, each type of rod-shaped particle has a width or diameter of less than 1 μm, the different types of particles are differentiable based on their length. In another example, 7 types of spherical silver particles make up an assembly. The different types of particles are differentiable based on their relative size. In yet another example, 8 types of rod-shaped particles, all composed of the same polymeric material, make up an assembly; although each type of rod-shaped particles have the same length, they are differentiable based on their diameter and/or cross-sectional shape.

The nanoparticles of this embodiment of the present invention may be functionalized as described above, and used in the same types of applications as the segmented nanobar code particles. In an assembly of particles, according to this

embodiment, the particle types may be, but are not necessarily composed of the same material.

A further example of an assembly of nanoparticles that fall within the scope of this embodiment of the invention is an assembly of particles, each type of which may have the same size and shape (with at least one dimension less than 1 μm) where the particle types are differentiable based on their composition. For example, an assembly of particles of the present invention may be comprised of 5 different rod-shaped nanoparticles of the same size and shape. In this example, the different types of particles are differentiable based on the material from which they were made. Thus, one type of nanorod is made from gold, another from platinum, another from nickel, another from silver, and the remaining type from copper. Alternatively, each particle type may contain a different amount of a dye material, or a different percentage of magnetizable metal. In each case, a given particle type would be differentiable from the other particle types in the assembly or collection.

Of course, this embodiment of the invention includes assemblies or collections in which combinations of size, shape and composition are varied. In preferred embodiments of the assembly of particles of this embodiment, all particle types have at least one dimension less than 10 μm and the particle types are differentiable, by any means, from the other particle types in the assembly. In this embodiment, the different types of particles may be functionalized and the differentiable characteristics of the type of particles encodes the nature of the functionalization. By encoding the nature of the functional unit, it is meant that the specific identifiable features of the nanoparticle can be attached selectively to a known functional unit, so that a key or log can be maintained wherein once the specific particle type has been identified, the nature of the associated functional unit is known.

In the case of metallic bar codes of approximately 100 nm or more in width and about 2 microns to 15 microns in length, in a preferred embodiment of this invention differences in metal segment reflectivities can be visualized using conventional light microscopy. Thus, it is possible to distinguish (and quantify) the number of rods by visual inspection. It is also possible to distinguish segments of different lengths within individual bar codes. Images have been obtained of a 9-striped bar code (Au-/Ag-/Au-/Ag-/Au-/Ag-/Au-/Ag-/Au) in which the four Ag segments were grown to different lengths. See **Figure 4**. The image was obtained using an optical microscope in reflected light mode, using a 400 ± 40 nm bandpass filter to improve resolution and enhance image

contrast. In addition to the visual and optical methods described above, detection and identification may be made by a multitude of different methods.

The image is interesting in several respects. First, it is clear that four distinct bright regions can be seen (which correspond to Ag segments). In this image, the apparent lengths (by microscopy) do not correspond to the estimated lengths. For example, the smallest bright segment does not appear to be one-tenth the length of the longest segment. This may be due to a non-linear current vs. length relationship, but more likely reflects physical limitations of the optics. The image was obtained with reflected light bright field microscopy. In this mode, the diffraction-limited optics give a theoretical resolution of about 2NA where NA = numerical aperture (in the system used to obtain these images, the resolution is about 400nm/2(1.4) = 143 nm). Thus, it is possible to distinguish two features as close together as 143 nm (Rayleigh criterion). Points closer together than this will appear as a single feature. However, note that an Ag stripe shorter than 143 nm is still visible under the microscope, since the Au sections separating it are longer than this distance. Thus, for a 2.5 micron bar code, one can easily imagine 12 stripes of 200 nm each, all of which are optically distinguishable. Alternately, it should be possible to create and "read" bar code rods of 2 micron having 10 stripes, with five segments of 150 nm and five segments of 50 nm.

The simultaneous bar code detection by reflectivity and analyte quantitation by fluorescence has also been demonstrated. Example 1 below describes such a result, and the images can be seen in **Figure 5**. Panel C shows a reflectivity image obtained at 400 nm that is used to identify each type of nanorod. In this case, the image shows a mixture of striped nanorods. Panel A, imaged at the wavelength of FITC emission, contains bright images of the first type of nanobar codes, and barely detectable ghosts of the second type of nanobar codes, which are easily subtracted digitally. In Panel B, imaged at the wavelength of Texas Red, the second type of nanobar codes show brightly, while the first type of nanobar codes are extremely dim.

The ability to read fluorescence and identify nanobar codes simultaneously comprise a powerful set of tools for multiplexed assays. In some embodiments, identification and detection can be achieved using the same signal. For example, a pattern of fluorescence can be used for identification while the intensity of the fluorescence indicates the concentration of the analyte. However, there are a large number of bioanalyses carried out by means other than fluorescence. Prominent among these is mass

spectrometry, rapidly becoming the tool of choice for detailed identification and analysis of polypeptides and proteins. There are two widely-used methods for biomolecular sample introduction in mass spectrometry: electrospray and matrix-assisted laser desorption/ionization (MALDI). In MALDI, the analyte of interest is embedded into a solid ultraviolet-absorbing organic matrix that vaporizes upon pulsed-laser irradiation, carrying with it the analyte. (See, e.g., Karas *et al.*, Anal. Chem. 60, 2299-2301 (1988)). During this process the energy absorbed by the matrix is transferred to the analyte that is ionized. The gas phase analyte ion is then sent to the Time-Of-Flight (TOF) mass analyzer. MALDI-TOF is currently successfully utilized for the analysis of proteins, polypeptides and other macromolecules. Even though the introduction of an organic matrix to transfer energy to the analyte has advanced tremendously the field of desorption mass spectrometry, MALDI-TOF still has some limits. For instance, the detection of small molecules is not practical because of the presence of background ions from the matrix. Also, MALDI experiments are inherently sensitive to matrix choice: matrix type as well as matrix amounts must often be tailored to the nature of the analyte (a severe limitation to the analysis of complex mixtures).

Recently, Sunner et al. have introduced the term SALDI for Surface-Assisted Laser Desorption/ Ionization (Sunner *et al.*, Anal. Chem. 67, 4335 (1995)). This technique is matrix free, allows analysis of small organic molecules and yields performances similar to MALDI. Noble metal nanoparticles are a vastly superior choice for laser-based ionization, for two reasons. (i) Colloidal noble metal nanoparticles exhibit very large extinction coefficients in the visible and near IR. This contrasts with organic matrices. (ii) Irradiation of Au nanoparticles is known to lead to dramatic enhancements in electric field strength at the particle surface: this is the basis of surface-enhanced Raman scattering. This leads to increased ionization efficiencies. Moreover, combined with nanobar code technology, SALDI-MS becomes a powerful molecular fingerprinting tool.

Conventional light microscopy has been used to image the nanorods, and should allow for automated "decoding" of the bar code signature. Additionally, fluorescence microscopy has been used to quantify the level of binding of a biomolecule to the rod. The detection and readout can also be accomplished with custom instrument designs and sophisticated image analysis software that are capable of detecting and reading the code of each nanobar code, and quantifying the fluorescence from molecules bound to the nanorod. Additionally, this detection system allows for highly focused laser excitation of

the appropriate wavelength to enable laser-induced desorption of non-covalently bound molecules from the surface of each individual nanorod.

As discussed above, though the preferred embodiment involves reflectivity as the mechanism for particle identification and fluorescence as the sensor readout, reflectivity changes themselves could also be used, with a potentially large payoff, for sensing. The two relevant points illustrated by Pt and Au reflectivities, are that (a) at most wavelengths, the reflectivity is different (thereby providing a contrast mechanism) and (b) at about 600 nm, they have the same reflectivity (a reflectivity isosbestic). At 450 nm, the Pt stripes will appear brighter (more reflective) than Au, and that at 600 nm, the opposite is true. At an intermediate wavelength, there is a reflectivity isosbestic, where no contrast would be observed.

## IMAGE ANALYSIS

This embodiment of the invention describes an image processing package developed for analyzing microscope images of nanobar codes, particularly when associated with an assay that generates a fluorescence signal. The main functions of the package are to identify each nanobar in an image and to quantitate the overall fluorescence intensity. As will be explained later, the identification of a nanobar can follow along two general paths. Either the nanobars can be grouped by features in their intensity profiles using clustering algorithms, or alternatively, the nanobars can be classified against a known set of nanobar profiles.

As an example, a sandwich immunoassay would be set up as follows. A number of flavors of nanobar would each be separately conjugated to a unique antibody. The nanobar/antibody pairs would then be pooled together and then added to the test solution/serum/etc. Labeled secondary antibodies are then added. The nanobars are imaged from the bottom of the well using a standard bright-field inverted microscope at a magnification of 100X. The image is captured using a high speed, scientific-grade CCD camera which can capture high resolution (> 1000pix X 1000pix) images. Another image is then captured using a fluorescent filter set which allows those nanobars with Ab-analyte-Ab* to be imaged. The intensity of each nanobar in the image is now proportional to the amount of bound antigen, and therefore the concentration of that particular analyte in the solution.

An image processing solution appropriate for this type of immunoassay and other similar assays must meet the following criteria. It must handle multiple images, both in the sense of analyzing and coupling the bright field (BF) and fluorescent images (FL), and in the sense of amassing statistics from many BF/FL pairs. In addition, if more than two materials are used in the composition of the nanobar codes, multiple bright field images and different wavelengths could be stored for nanobar code identification purposes. As an example, consider a typical well in a 96 well plate. It has a useful imaging area of approximately ( 20 $mm^2$). Each BF/FL image represents approximately 0.01 $mm^2$ and approximately 50 nanobars can fit into each image without significant overlap. Therefore, theoretically, about 100,000 nanobars can be processed in each well of a 96-well plate. To capture and process these nanobars, the image processing solution needs to accumulate data on each flavor of nanobars in each image and cache this data to produce statistics on the total sample.

The typical size of each nanobar for immunoassays ranges from 5-10 μm long with widths nominally 200nm. In pixel units this measurement translates to approximately 50 pixels long by 4 pixels wide (each pixel represents approximately a 100 nm square and with a numerical aperture of 1.3 and a nominal wavelength of 500 nm, the blur spot is approximately 250nm). The object size is an important consideration when designing image processing software since it affects the choice of pattern recognition algorithms.

Locating a nanobar in an image and determining its "flavor" can follow a number of paths, as illustrated in **Figure 6**. Initially, it must be decided whether to adopt a rotational and scale invariant (within limits) algorithm that determines the flavor without regard to the nanobar orientation. Alternatively, the orientation of the nanobars can be determined first and the recognition of nanobars can follow one of two secondary paths. Either the software can group or cluster those nanobars with a similar intensity profile along their long axis, or, since the flavors of nanobars are known *a priori*, the software can take a classification approach, whereby the software finds the best correlation between a library of known nanobar profiles and those nanobars found in the image. The former method can use clustering algorithms that map the intensity profile into a multidimensional vector based on particular features known to be characteristic of nanobars. The latter approach includes neural networks and/or straight correlation algorithms. Neural networks have the advantage that they are learning-based networks that would allow the network to adapt to a preferred state based on initial classification

feedback from the user. Since the user has *a priori* knowledge of the ratio of nanobar flavors, clustering algorithms would have the advantage that the system could eliminate those rods that don't fall into a cluster with a given relative size ratio.

For simplicity sake, the preferred embodiment of the nanobar recognition software first identifies the orientation and then classifies using correlation. However, whichever algorithm is chosen, it needs to handle the fact that nanobars can overlap, especially in dense conditions. Nanobars can be found to be broken as well as vary slightly in length and width.

The primary flowchart for this embodiment of the nanobar code software is given in **Figure** 7. The program assumes a series of brightfield/fluorescent (BF/FL) image pairs whereby each pair is stored in an SM1 custom binary format in a given location with a predefined naming convention to match those image files belonging to a single well. The user chooses the first image pair in the series.

The first BF/FL image pair is read and stored in memory as two separate images. Processing begins with the bright-field image. As shown in **Figure 7**, a high pass filter is first applied to the brightfield image. This kernel has the effect of both enhancing the edges of nanobar codes and separating those nanobar codes that are close in proximity. Then the image noise is first assessed using a peak-peak noise algorithm. (See, e.g., United States Patent No. 5,556,764 of Sizto *et al.*, incorporated herein by reference.) The image noise, combined with the median background-level are well known in the field for determining the threshold level that must be applied to the image for segmentation, a method of isolating objects in a binary image. The image is then thresholded at a level, *Thresh*, given by the sum of the background level and the noise level multiplied by a user-defined factor, ThreshFactor, i.e.,

$$\text{Thresh} = \text{BgndLevel} + \text{P-P\_Noise} * \text{ThreshFactor}. \qquad (1)$$

The result of thresholding is a binarized image, where it is assumed ideally that those nonzero pixels correspond to nanobars and the zeroed pixels correspond to background. To test this assumption, segmentation, thinning, and a number of conditions are applied. The segmentation algorithm applies an 8-point connectivity rule to isolate regions of pixels in contact. Each region is indexed and treated separately in the following processes.

Each indexed region or "blob" is first tested for size in terms of the number of pixels. If this exceeds a user-defined threshold, the blob is discarded, on the assumption that it is either an artifact or a group of nanobars in contact. If the blob meets the size criteria, it is thinned. Thinning or skeletonization is an important aspect of the present nanobar processing software. There are a variety of thinning algorithms known to those skilled in the art, each with varied conditions on how to conditionally erode blobs in order to leave a skeletal (or stick-figure) representation of the shape. If the proper algorithm is not chosen, the thinned blobs, representing the long-axis of the nanobar, can contain numerous artifacts such as breaks, branches, and loops. It was found that a combination of thinned algorithms, the Zhang/Suen/Stentiford/Holt algorithm is best suited to thinning nanobar images.

Following the thinning of a particular blob region, the size is then rechecked against another user-defined threshold. Thinned nanobars that are too short are discarded. Another condition to be tested on the thinned nanobar image is the number of branches. Multiple branches can represent overlapping nanobars. The software can discard these, or re-index them as separate nanobars, each branch being a different nanobar. The present implementation discards thinned blobs with multiple branches.

At this point in the program the thinned representation of the nanobar is fitted to a line. The line fit can be implemented using a least squares fitting algorithm in polar coordinates (to eliminate vertical and horizontal line issues). The residual of the line fit determines the quality of the fit. If the residual exceeds a user-defined threshold, the blob is discarded.

The localized line is used to profile the intensity level of the nanobar in the original bright-field image. The theoretical pixels of the fitted line, which can be fractional, are determined by two conditions. The localized theoretical line should be centered at the same center-of-mass as the original thinned line, and the number of theoretical pixels should be set to the same number as the thinned image. Additionally, the user can define a line thickness, such as four pixels (the average pixel width of a nanobar), which are averaged perpendicular to the line to reduce intensity noise in the profile.

The intensity profile is then correlated to all the pre-defined nanobar flavors. These predefined flavors are stored in a relative intensity fashion in such a way that the brighter metal represents a higher quantity, and the less intense metal, a lower quantity. In one embodiment, silver and gold were used with silver having higher reflectance at the

wavelengths of interest. Thus, a three-striped bar that is comprised of only two metals, such as silver and gold, could be represented in a simple binary fashion as 101 (i.e., silver, gold, silver). This 101 vector would be expanded to the same number of pixels found in the thinned profile of the nanobar. Each flavor is expanded in this same way and a direct correlation is performed against each flavor. The highest correlation value represents the proper nanobar flavor.

Once a nanobar has been identified, the fluorescent intensity of the nanobar is assessed using the stored fluorescent image. More specifically, those same pixel coordinates that are used to highlight the nanobar image in the brightfield segmented image are used to quantitate fluorescent intensity. Difficulties that must be addressed include image offset between the brightfield/fluorescent pair, method of assessing *average* fluorescent intensity, and possible loss of pixels involved in fluorescent intensity. The first challenge, image offset, can be remedied for those cases where the image offset is know and static. Switching filters between brightfield and fluorescent images in the optical path results in a static offset which can be determined by the user and adjusted for in determining fluorescent intensity. Methods to assess average fluorescence are complicated by the fact that fluorescent intensity can appear "blotchy" along a rod. One method that can be adopted is the sorting and averaging of the top $N$ pixels in a nanobar pixel area. Other histogramming techniques can be used to reduce intensity noise among a population of uniformly labeled rods.

Finally, the correlations and intensity values are stored for each rod and the next image in the batch is analyzed. Once all images have been analyzed, the data for each flavor of nanorod is combined to give intensity statistics for each flavor of nanorod over many images composing a well. This information is written out to a text file.

In an additional embodiment, the solution for thresholding is to determine a local background level since the background image intensity can vary over the expanse of the image. This algorithm is complicated by the fact that clusters of nanobars can occur in portions of the image, thereby making the distinction between background intensity and nanobar intensity difficult. A means of circumventing this problem is dynamic thresholding, whereby the threshold is varied and segmentation and thinning are repeatedly applied. If the threshold was too high, few thinned segments would be found and if the threshold was too low, the number of total branches in the image would be high.

MLSC IMAGING

In certain embodiments of the invention, rods are imaged in a microscope with a high NA (>1.3) objective. A solution of rods is placed in a glass capillary or a micro-well. The rods are non-colloidal and so quickly settle to the bottom of the container. The microscope is focused onto the bottom of the well and a digital camera is used to acquire a reflectance image and then a corresponding fluorescence image. The reflectance image is analyzed to find the rods and identify them. Typically it is assumed that the rods in a fluorescence image are in the same location. Basic histogram analysis is performed on the pixels in the fluorescence image to calculate the average fluorescence of a rod. The area CCD used in such embodiment can be slow to read out and read out speed decreases with increasing detector size (and thus number of rods analyzed per image). Furthermore, the fluorescence sensitivity of the wide field system is less than ideal and there is no opportunity to reject background fluorescence.

In a further embodiment of the invention a Micro Volume Laser Scanning Cytometer or a MLSC instrument is used to generate fluorescence images of the rods in the sample. The standard MLSC instrument is modified with a linear CCD array detector to obtain a reflectance image while the fluorescence image is obtained. A real-time focusing servo is also added to keep the sample in focus at all times during image collection.

MLSC technology is described in United States Patent Numbers 5,547,849 and 5,556,764 and in Dietz *et al.*, Cytometry 23:177-186 (1996); United States Patent Application Serial No. 09/378,259, filed August 20, 1999, entitled "Novel Optical Architectures for Microvolume Laser-Scanning Cytometers," and International Application PCT/US00/11133, filed April 26, 2000, entitled "System for Microvolume Laser Scanning Cytometry," each of which is incorporated herein by reference in their entirety.

The advantages of this system are as follows:

a) The MLSC instrument provides a method to reject background and so improve fluorescence sensitivity.

b) A laser is used to excite fluorescence and so high excitation power densities are available, further improving sensitivity.

c) Linear CCDs can be read out much faster than area CCDs and are much less expensive.

d) The reflectance and fluorescence images are obtained simultaneously providing exact image registration.

The schematic of an MLSC apparatus for imaging nanobar codes according to the present invention is shown in **Figure 8**. Collimated excitation light from a Helium Neon Laser is deflected by the dichroic excitation filter, HeNe Dichroic. Upon reflection, the light is incident on the scan mirror, Galvo Mirror. The scan mirror is attached to a galvanometer which can rapidly oscillate the mirror over a fixed range of angles. Next, 2 relay lenses GRL1 and GRL2, image the scan mirror onto the entrance pupil of a microscope objective. This optical configuration converts a specific scanned angle at the mirror to a specific field position at the focus of the microscope objective. The spot diameter, which sets optical resolution, is determined by the diameter of the collimated beam and the focal length of the objective.

Fluorescence samples placed in the path of the swept excitation beam emit stokes shifted light. This light is the collected by the objective and collimated. The collimated light emerges from the 2 relay lenses still collimated and impinges upon the scan mirror which reflects and descans it. The stoke shifted light now passes through the HeNe Dichroic. The long pass filter F2 rejects any excitation light that leaks through the HeNe dichroic.

The system can detect 2 different emission colors. Fluorescence emission is passed by the HeNe dichroic and focused onto Aperture 1 and then further parsed to 2 PMTS by the Fluorescence Dichroic based on their relative wavelengths, bluest to PMT1 and reddest to PMT2. The aperture rejects any light that is out of the plane of best focus at the sample. More fluorescent colors can be detected by adding more PMT pairs, lenses, apertures and dichroic filters.

The reflectance image is obtained with separate optical paths. A light source is used to generate a line of illumination. This source could be an arc lamp, incandescent lamp, metal halide lamp, LED or laser. An example would be a metal halide lamp with a filter to pass wavelengths from 400 nm to 450 nm, the optimal for Au, Ag and Ni contrast. A second lens, L5, is used to collimate the lamp output and a cylindrical lens, CYL 1, in conjunction with the objective, creates line illumination at the sample. The illumination passes through a partial silvered mirror PSML1 and then reflected by the Image Dichroic. The Image Dichroic separates the red fluorescence light from the blue reflectance light. The objective then focuses a line of light onto the sample. Collected reflected light from

the rod sample is collimated by the objective and again reflected by the Image Dichroic. The reflectance light passes through the mirror PSM 1and is re-imaged on the linear CCD detector by the Tube Lens.

The sample, glass capillary or micro well plate, is mounted on a XY stage. While the galvanometer driven mirror scans the excitation beam in Y the sample is moved in X at a constant velocity. The emitted fluorescence photon flux is converted to an electronic current by the PMTs. The currents are converted to voltages by a pre-amplifier in the detection electronics. The voltages are sampled at regular intervals by an analog to digital converter. The sample interval times the swept beam rate determines the pixel spacing in Y, the fast scan direction. The subsequent line rate times the X stage scan speed determines the pixel spacing in X. The corresponding reflectance image photons are converted to electronic charge by the linear CCD. The output of the CCD is passed to a third A/D for digitization and storage in the control computer.

The XY stage not only scans but shuttles samples so many samples can be scanned sequentially by computer control.

The microscope objective is mounted on a high precision servo drive, piezo-electric or other. The microscope objective is moved up and down to correct focus while scanning. The focus correction signal is provided by the reflected HeNe light. Reflected HeNe light traces it path back to a beam splitter. The split reflected light is focused by Lens 4 through Aperture 2 and onto a detector. The signal at this detector is brightest when the laser is focused onto the water glass interface of the sample chamber. The aperture size and lens focal length are chosen to provide a very sharp drop in signal as the sample is moved out of focus. Since all rods are on the bottom this corresponds with best image focus. The signal from the focus detector is sent to a control circuit which converts signal changes into position.

**FLOW IMAGING**

Because of the huge number of different flavors of nanobar codes that can be made, there needs to be means to rapidly and accurately image nanobar codes. A typical barcode may be 200 nm in diameter and 8 μm long. If one wanted 20 different stripes, stripes that are 400 nm wide are preferred. As the multiplicity of the assay increases so do the number of rods that must be imaged and analyzed. The technical challenge is to image the bar codes rapidly and at high resolution. One needs a numerical aperture >1.0 to

resolve 400 nm features. Such a high numerical aperture has < 1 μm depth of focus and so the beads must be kept in a localized area when imaged.

Nanorods provide a method for highly multiplexed assays, however, they must be analyzed in a rapid fashion by a high resolution imaging system. These two requirements pose some challenges since it means the rods need to be tightly localized. Imaging time must be spent on the rods and not on any background and so they must be localized in the imaging systems field of view. Furthermore, a high lateral resolution imaging system comes with very narrow depth of focus so the rods must be localized in the imaging systems focal plane.

In this embodiment of the invention, nanorods are injected into a sheath flow system. Flow systems like this exist for cell analysis, i.e. Flow Cytometers. It is known that particles flowing in a capillary are localized to the center of the flow region and they orient themselves longitudinally to the flow direction. As the rods flow they first encounter a trigger to let the digital imaging system know a rod is coming. Next the digital imaging system takes an image of the nanorod. The image is piped into a Digital Signal Processor for rapid analysis to determine the nanorod sequence. Thus the nanorod is characterized. Once characterized the rod can be piped to multiple assay paths. If a fluorescence assay is performed the rod flows past an excitation laser beam and filtered fluorescence is detected. If Mass Spectrometry is to be performed then the rods are injected into a Mass-Spectrometer.

See **Figure 9** for a graphic depiction of the invention. The contents of a well of nanorods are injected into an optically clear capillary. A sheath flow system as used in flow cytometry is used to localize the particles to the middle of the flow stream. A sheath flow system can localize particles to a diameter of 10 microns or less. Smaller dimension capillaries may be used to localize the particles to the center of the flow stream without using sheath flow but they might limit flow rate. One geometry would be to use a large channel or capillary at the inlet or outlet that necks down to a narrow region where the particles are analyzed. The flow rate is set so that up to 1000 particles/sec pass by the detector. If a particle is ~ 10 μm long then the flow particle velocity would be ~ 10 microns/particle x 1000 particles/sec=10 mm/sec to 100 mm/sec particle flow velocity.

The read region consists of two optical paths. The first is an optical trigger. A laser beam is finely focused so that its waist is in the center of the flow field. The light is allowed to expand and impinge upon a detector. When a particle passes this beam it

breaks the light and the detector senses the break. This signal is sent to the digital imager to signal the arrival of the particle and the start of the digital imager readout. An appropriate delay would be in place to account for the transit time from trigger beam to imaging region.

Next down stream is the imaging system. The flow field is illuminated with an incandescent light source, using epi-brightfield illumination. A very high numerical aperture, > 1, microscope objective is used to image particles in the exact center of the flow field on to a digital imaging device. The digital imaging device may be a linear array detector or area detector. The linear array detector would be clocked to read successive image lines as the particle passes by the field of view. An electronic controller stitched together the image and store it or process the image in real time to determine the code in the nanorod. An area detector requires a short exposure time, 1 to 10 microseconds, followed by a read out. Progressive scan area detectors may perform this function.

Nanorods are typically 200 nm in diameter and 5 to 10 microns long. Bar code stripes would be 400 nm or greater. The small size of the nanorods requires a high resolution imaging system. Imaging lens of numerical aperture > 1 is needed. A imaging system of this speed has very narrow depth of focus, i.e., depth of focus ~ wavelength, or 500 nm for imaging with a 500 nm light source. This can be dealt with in one of two ways. The first is to constrain the particles to the middle of the flow stream. Sheath flow technology can only get the particles within a 5 to 10 micron band. Small capillaries could be used but would lead to flow rate restrictions. Wave front encoding optics are used to extend the depth of focus of imaging systems. Digital optics are placed in the imaging optical train, the collimated light path of an infinite conjugate imaging system. The optics extend the depth of focus by as much as 10 times at the expense of sensitivity. Wavefront optics also require an extra image processing step. Images could be processed via a specialized DSP or off line. Alternatively, an excess of rods may be used and some rods will flow by and be out of focus. They would be imaged but the code could be ambiguous. These particles could be ignored or later assay information could be used to determine their rod population.

After the imaging systems particles can then be assayed using a fluorescence detection system similar to that used in flow-cytometry. The particles pass through a large laser spot. Excited fluorescence is collected by a high NA lens and re-imaged, through the

appropriate filters onto a PMT or other detector. Filters may be replaced with a spectrograph and a CCD detector could be used.

## EXAMPLES

### EXAMPLE 1

A solution based sandwich immunoassay has been developed for use on bar code rods that employs optical microscopy fluorescence detection. The assay has been performed on Au, Au/Ag and Au/Ni rods of varying segment patterns. The nanobar code is read based on differences in reflectivity of the metals at differing wavelengths. **Figure 5** depicts the results of this experiment.

Initially, the sandwich immunoassay was performed on two types of rods, Au/Ag and Au rods, using the following system: anti-rabbit IgGFc/rabbit IgG/anti-rabbit IgGH&L labeled with Texas Red. Fluorescence images have been taken with filters for FITC on a mixture of rods and the rods appear to be the same metal composition with a 600 nm bandpass filter, however, changing to a 400 nm bandpass filter reveals the bar code ID.

Then two different sandwich immunoassays were performed on two different types of bar code rods. For this experiment the same Texas Red (TR) assay as mentioned above was used along with the following system: anti-human IgGFc/HIgG /anti-human IgGg specific. FITC images were taken first since this fluorophore photobleaches much quicker than TR. Since it was established that at least two fluorophores could be distinguished, a simultaneous solution based assay was attempted next. The rods were derivatized with the capture antibody in separate tubes after which they were mixed together for completion of the assay in order to mimic conditions present in serum samples. Two fluorophores were necessary to determine the amount of non-specific binding as well as cross-reactivity. Initially, there was a significant amount of cross-reactivity between the two systems as well as some non-specificity to the rod surface. To circumvent this problem, an amino terminated PEG was used, which significantly cut down on non-specificity, and BSA was used to help with cross-reactivity. The simultaneous, solution based two-system sandwich immunoassay was successfully completed. 4μm Au/Ag/Au rods were derivatized with a-human IgG, (FITC); and 8μm Au/Ni/Au were derivatized with a-rabbit IgG (TR). The Au sections of the Au/Ni/Au rods were selectively derivatized as evidenced by the lack of

fluorescence on the Ni sections. Furthermore, Ag appeared to enhance fluorescence from FITC.

To investigate the enhancement factors of Ag with FITC, a sandwich assay was performed using two different fluorophores on the same type of rods. The human IgG FITC system and a new system of the following: anti-Cytochrome c/biotinylated Cc/streptavidin-phycoerythrin (PE) was used. As for the human IgG system, there was brighter fluorescence on sections of the rod which correspond to the Ag sections, as revealed in the reflectivity image. However, no enhancement from Ag was seen for the PE system. Thus, it is likely that the enhancement is a wavelength specific phenomena, both with respect to the fluorophore absorbance and the nanobar code extinction (absorbance and scattering).

**EXAMPLE 2**

Flow cytometry experiments have been employed to quantitate fluorescence from immunoassays or nanobar codes. Both human IgG and biotinylated Cc systems have been investigated. The rabbit IgG system was switched to the biotinylated Cc system because TR could not be excited with 488 nm in the flow cytometry instrument. Titration curves were prepared for the human IgG and the biotinylated Cc systems on Au-/Ag nanobar code. From the graphs, it appears that the titration curve for human IgG contains an inflection point, whereas the biotinylated Cc system does not. Instead, it reaches a maximum and appears to level off. The shape of the curve for the human IgG system may originate from Ag enhancement of FITC. Flow cytometry experiments may be conducted to determine the amount of antibody binding capacity (ABC), as well as the concentration of capture antibody needed to optimize the system.

**EXAMPLE 3**

The use of colloidal Au or Ag for detection of bioassays has been studied. This relates to the differences in reflectivity of metals at different wavelengths. In theory, the bar code ID, or portions thereof, would not be visible at the reflectivity isobestic , i.e. about 600 nm for Au and Ag. However, selectively placing colloidal particles on all or part of the bar code would result in a reflectivity change, hence a reflectivity contrast. Colloidal Au particles are best for this aspect since they are easier to make monodisperse, derivatize, and biocompatible. A preliminary experiment has shown that adsorption of a

layer of Ag colloid can alter the reflectivity of Au/Ag rods. This was accomplished by adsorbing a monolayer of 1,6-hexane dithiol on the rods followed by exposure to Ag colloid. TEM data confirms binding of colloidal Ag to the nanobar codes. At 400 nm, the characteristic striped pattern of reflectivity can be seen with or without addition of colloidal Ag. However, at 600 nm, the striping pattern cannot be seen in the absence of Ag nanoparticles but can be seen in the presence of Ag nanoparticles.

These data indicate that there is a differential electromagnetic interaction between the Ag nanoparticles and the Ag and Au segments of the nanobar code, since the TEM data indicate a uniform distribution of the Ag material over the surface of the nanobar code. Note that the changes in reflectivity do not need to involve an isosbestic (i.e., from no differential reflectivity to differential reflectivity or visa versa). All that is required is that a chemical or biochemical event be coupled to a change in reflectivity. Moreover, this change in reflectivity does not need to be different for the various segments. Thus, the most general implementation involves a molecular binding/debinding induced change in reflectivity of one or more segments for the entire nanobar code. A more specific embodiment involves changes in reflectivity leading to elimination (or generation) of a reflectivity isosbetic.

**CLAIMS:**

We claim:

1. A method for identifying a segmented nanoparticle wherein the segments of said nanoparticle may be differentiated by reflectivity, comprising:

obtaining the reflectance image of said nanoparticle;

identifying said nanoparticle in said image by distinguishing from background;

identifying the reflectivity pattern of said nanoparticle; and

correlating said pattern with preset nanoparticle profiles to identify said segmented nanoparticle.

2. The method of claim 1 wherein said segmented nanoparticle has a length from 10 nm to 50 μm and the nanoparticle width is from 5 nm to 50 μm.

3. The method of claim 2 wherein said segmented nanoparticle is comprised of 2-50 segments, wherein the particle length is from 1-15 μm, the particle width is from 30 nm to 2 μm, and the length of said segments is from 50 nm to 15 μm.

4. The method of claim 1 wherein at least one segment of said nanoparticle is comprised of material selected from the group consisting of: a metal, any metal chalcogenide, a metal oxide, a metal sulfide, a metal nitride, a metal phosphide, a metal selenide, a metal telluride, a metal antimonide, a metal alloy, a semiconductor, a semi-metal, any organic compound or material, any inorganic compound or material, any organometallic compound or material, a particulate layer of material, and a composite material.

5. The method of claim 1 wherein said reflectance image is obtained in a MLSC apparatus.

6. The method of claim 1 wherein said nanoparticle is in a flow system when the reflectance image is taken.

7. A method for simultaneously identifying a nanoparticle and detecting an assay result wherein said assay is associated with said nanoparticle and wherein the nature of said assay is encoded by said nanoparticle, comprising:

identifying the nature of said nanoparticle;

decoding said nanoparticle so that the nature of said assay is identified; and

simultaneously detecting said assay result.

8. The method of claim 7 wherein said nanoparticle has a length from 10 nm to 50 μm and the width is from 5 nm to 50 μm.

9. The method of claim 8 wherein said nanoparticle is comprised of 2-50 segments, wherein the particle length is from 1-15 μm, the particle width is from 30 nm to 2 μm, and the length of said segments is from 50 μm to 15 μm.

10. The method of claim 7 wherein at least one segment of said nanoparticle is comprised of material selected from the group consisting of: a metal, any metal chalcogenide, a metal oxide, a metal sulfide, a metal nitride, a metal phosphide, a metal selenide, a metal telluride, a metal antimonide, a metal alloy, a semiconductor, a semi-metal, any organic compound or material, any inorganic compound or material, any organometallic compound or material, a particulate layer of material, and a composite material.

11. The method of claim 7 wherein said identification of said nanoparticle is done using a technique selected from the group consisting of optical detection mechanisms, scanning probe techniques, electron beam techniques, electrical techniques, magnetic techniques, and mechanical techniques.

12. The method of claim 11 wherein said optical detection mechanisms is selected from the group consisting of absorbance, fluorescence, Raman, hyper Raman, Rayleigh scattering, hyperRaleigh scattering, CARS, sum frequency generation, degenerate four wave mixing, forward light scattering, back scattering and angular light scattering.

13. The method of claim 11 wherein said scanning probe technique is selected from the group consisting of near field scanning optical microscopy, AFM, STM, chemical force microscopy and lateral force microscopy.

14. The method of claim 7 wherein said assay detection is accomplished by fluorescence detection.

15. The method of claim 7 wherein said assay detection is accomplished by mass spectrometry detection.

16. A method for identifying a specific type of segmented nanoparticle wherein said type encodes some information and wherein said nanoparticle has a particle length from 1-15 μm, the particle width is from 30 nm to 2 μm, and the length of said segments is from 50 nm to 15 μm, comprising:

identifying the specific nanoparticle; and

decoding said nanoparticle to obtain said information.

17. The method of claim 16 wherein at least one segment of said nanoparticle is comprised of material selected from the group consisting of: a metal, any metal chalcogenide, a metal oxide, a metal sulfide, a metal nitride, a metal phosphide, a metal selenide, a metal telluride, a metal antimonide, a metal alloy, a semiconductor, a semi-metal, any organic compound or material, any inorganic compound or material, any organometallic compound or material, a particulate layer of material, and a composite material.

18. The method of claim 16 wherein said identification of said nanoparticle is done using a technique selected from the group consisting of optical detection mechanisms, scanning probe techniques, electron beam techniques, electrical techniques, magnetic techniques, and mechanical techniques.

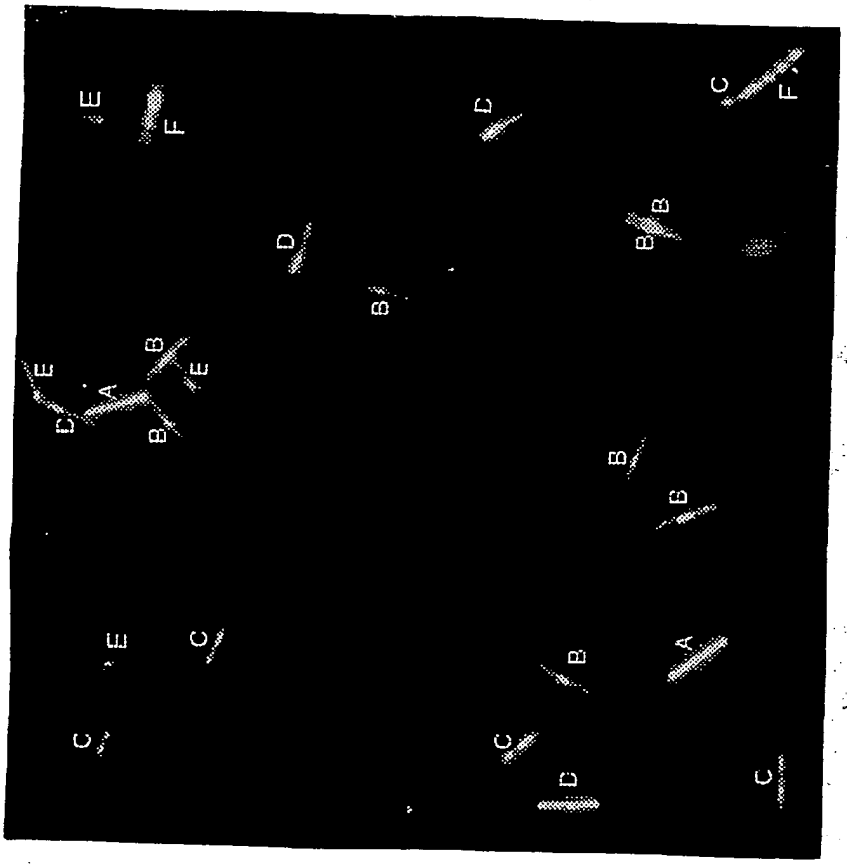
19. The method of claim 16 wherein said optical detection mechanisms is selected from the group consisting of absorbance, fluorescence, Raman, hyper Raman, Rayleigh

scattering, hyperRaleigh scattering, CARS, sum frequency generation, degenerate four wave mixing, forward light scattering, back scattering and angular light scattering.

20. The method of claim 16 wherein said scanning probe technique is selected from the group consisting of near field scanning optical microscopy, AFM, STM, chemical force microscopy and lateral force microscopy.

21. The method of claim 16 wherein said assay detection is accomplished by fluorescence detection.

22. The method of claim 16 wherein said assay detection is accomplished by mass spectrometry detection.



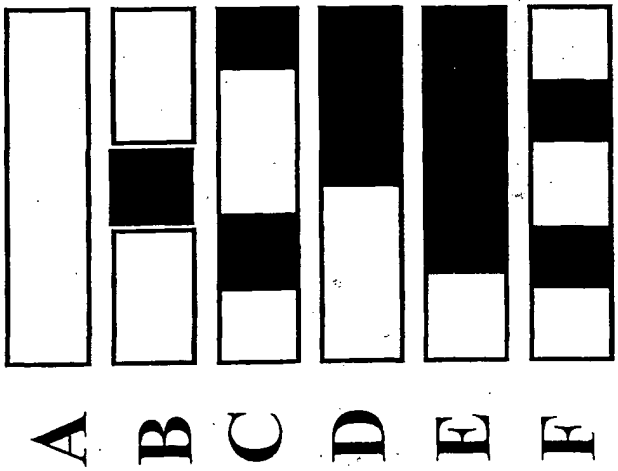



Fig. 1

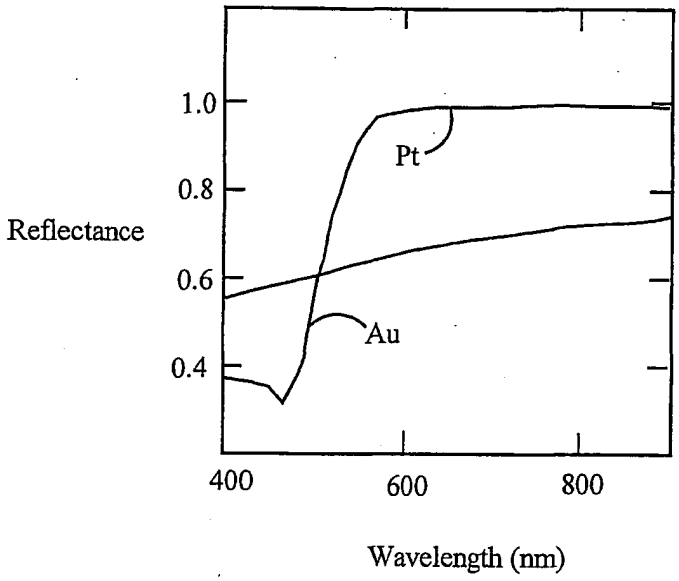
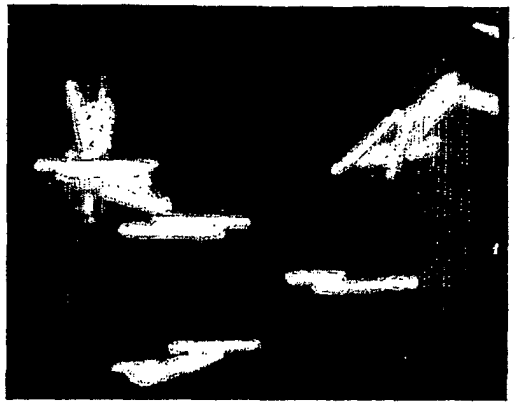
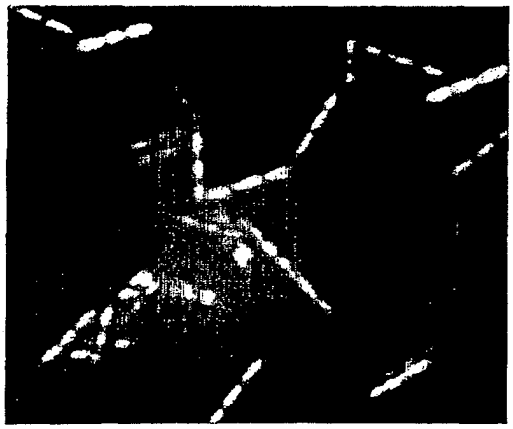



Fig. 2



600 nm

Fig. 3B



400 nm

Fig. 3A

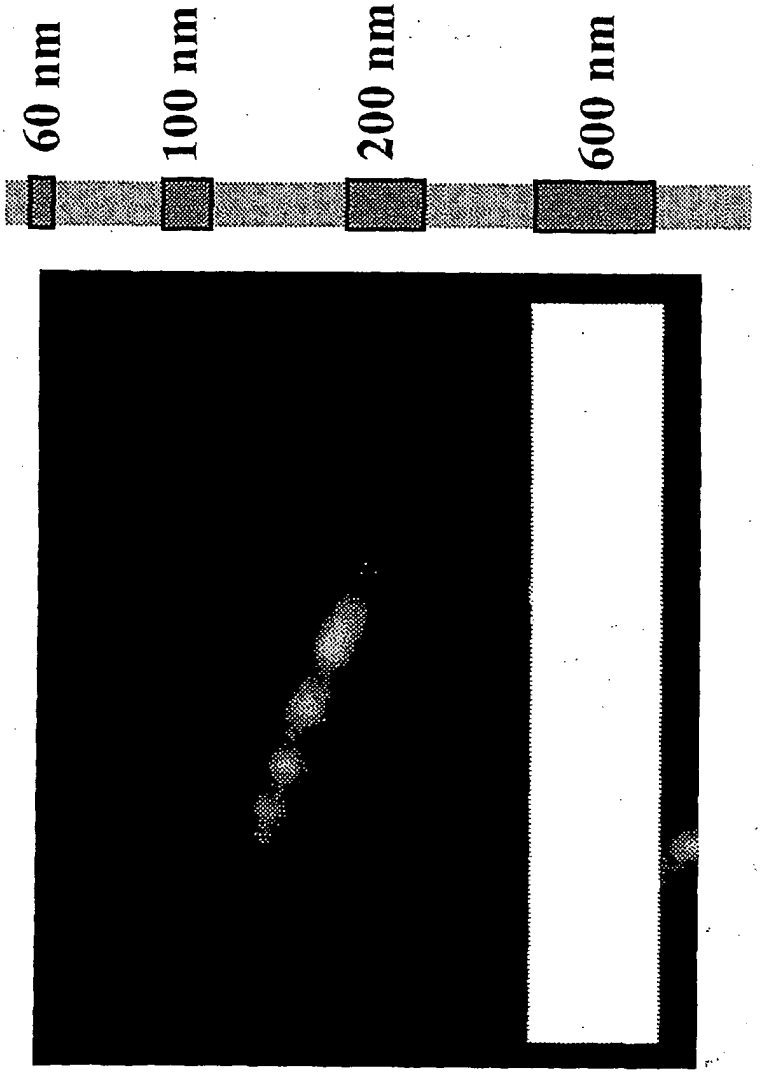



Fig. 4

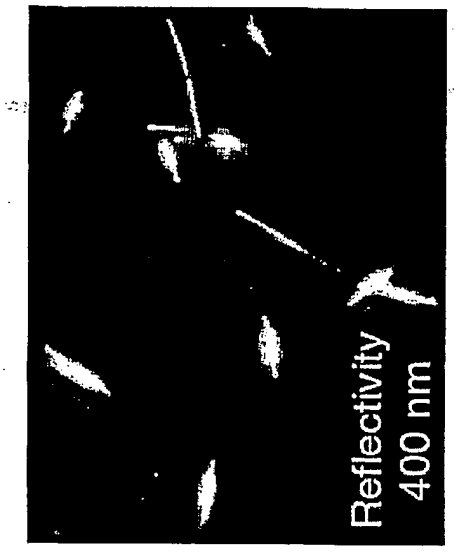



Fig. 5C

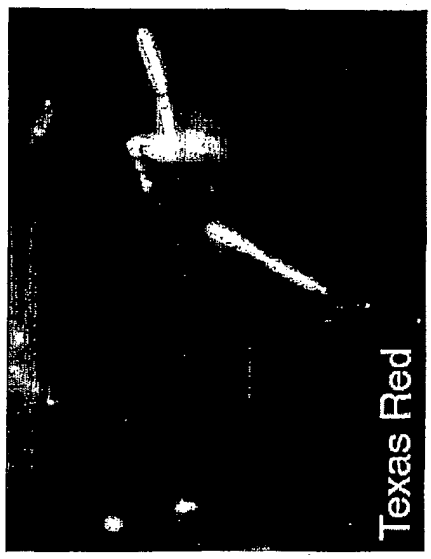



Fig. 5B

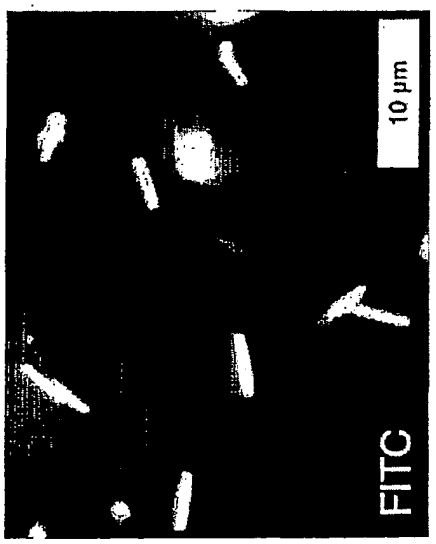
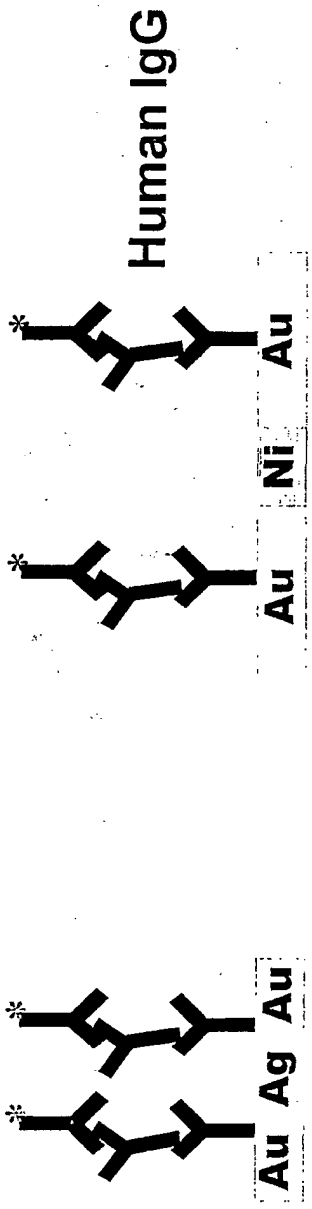



Fig. 5A



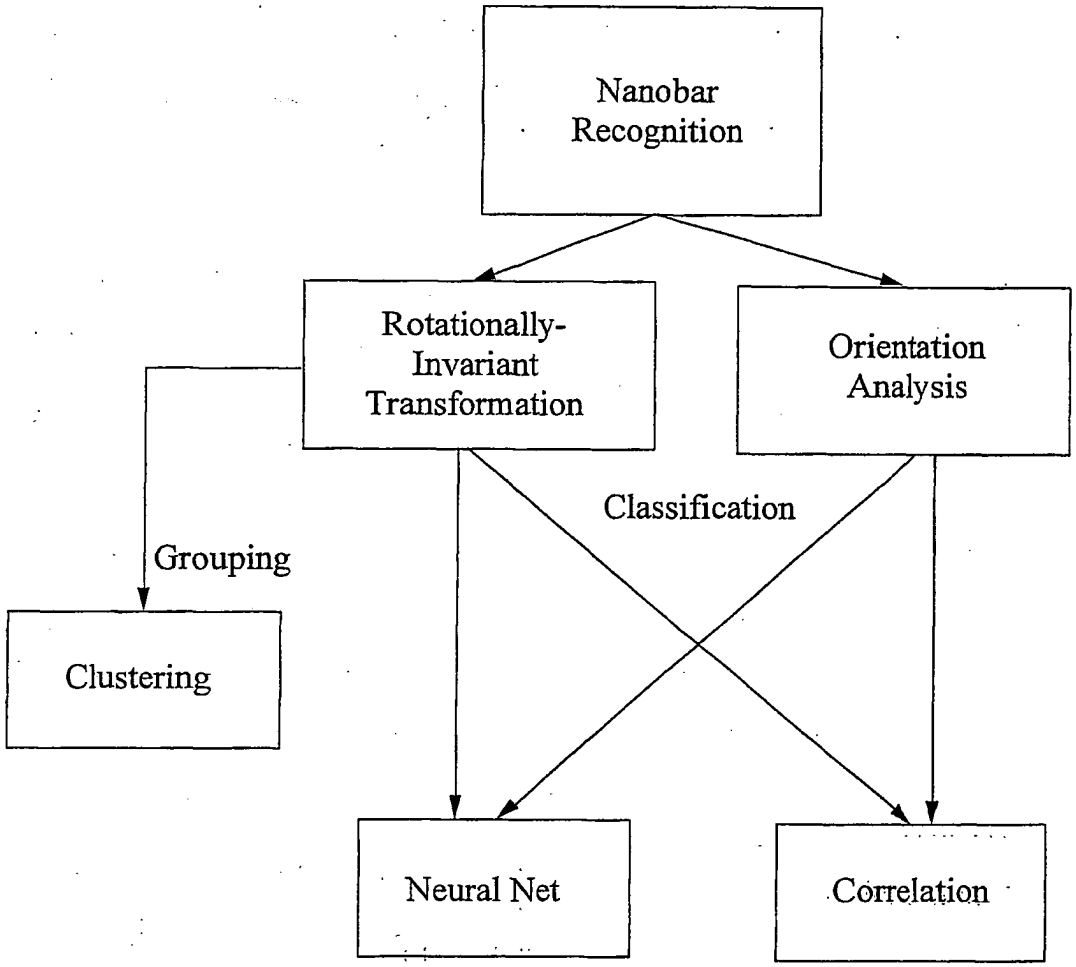

Nanobar Recognition
Rotationally-Invariant Transformation
Orientation Analysis
Grouping
Classification
Clustering
Neural Net
Correlation

Fig. 6

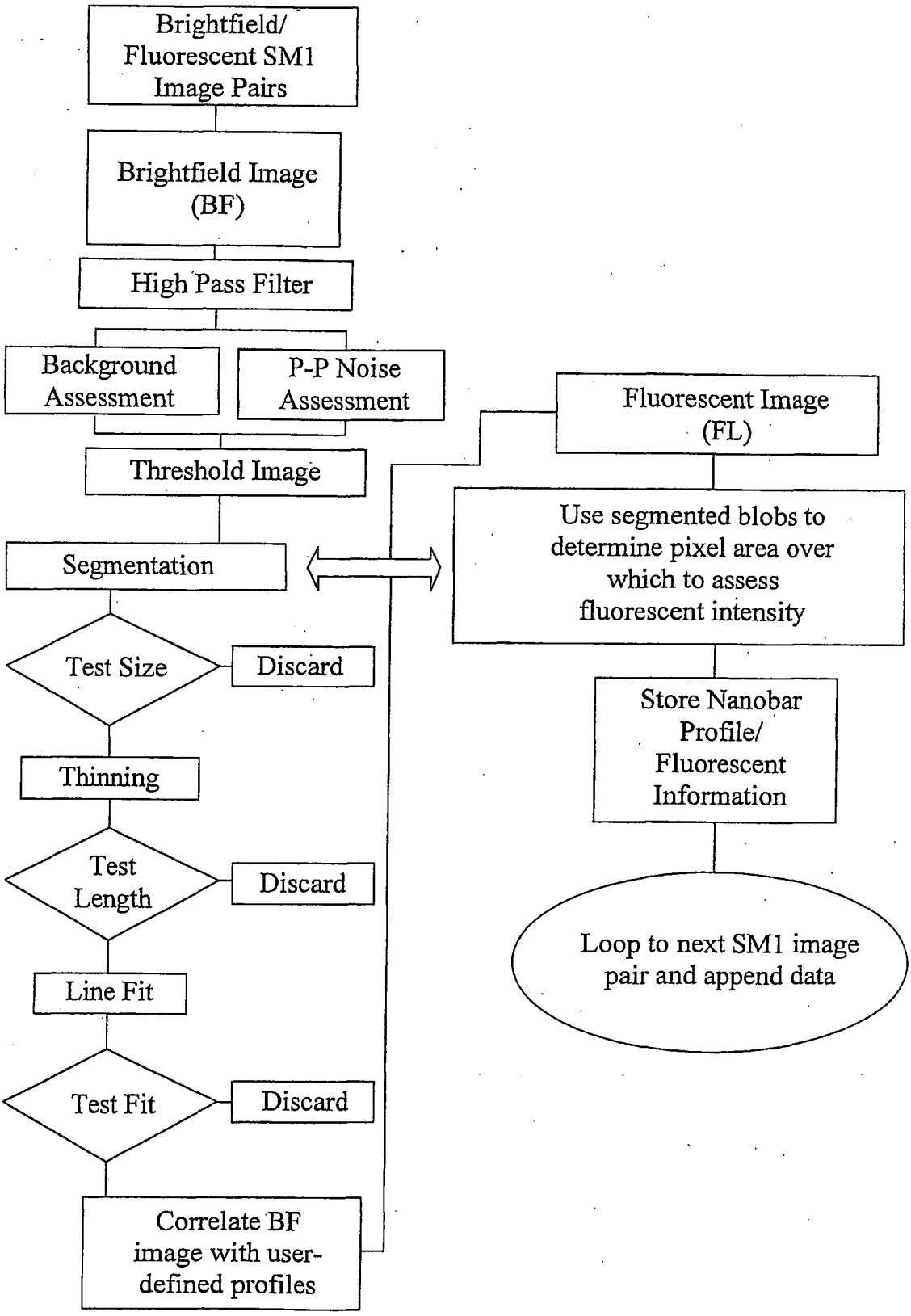
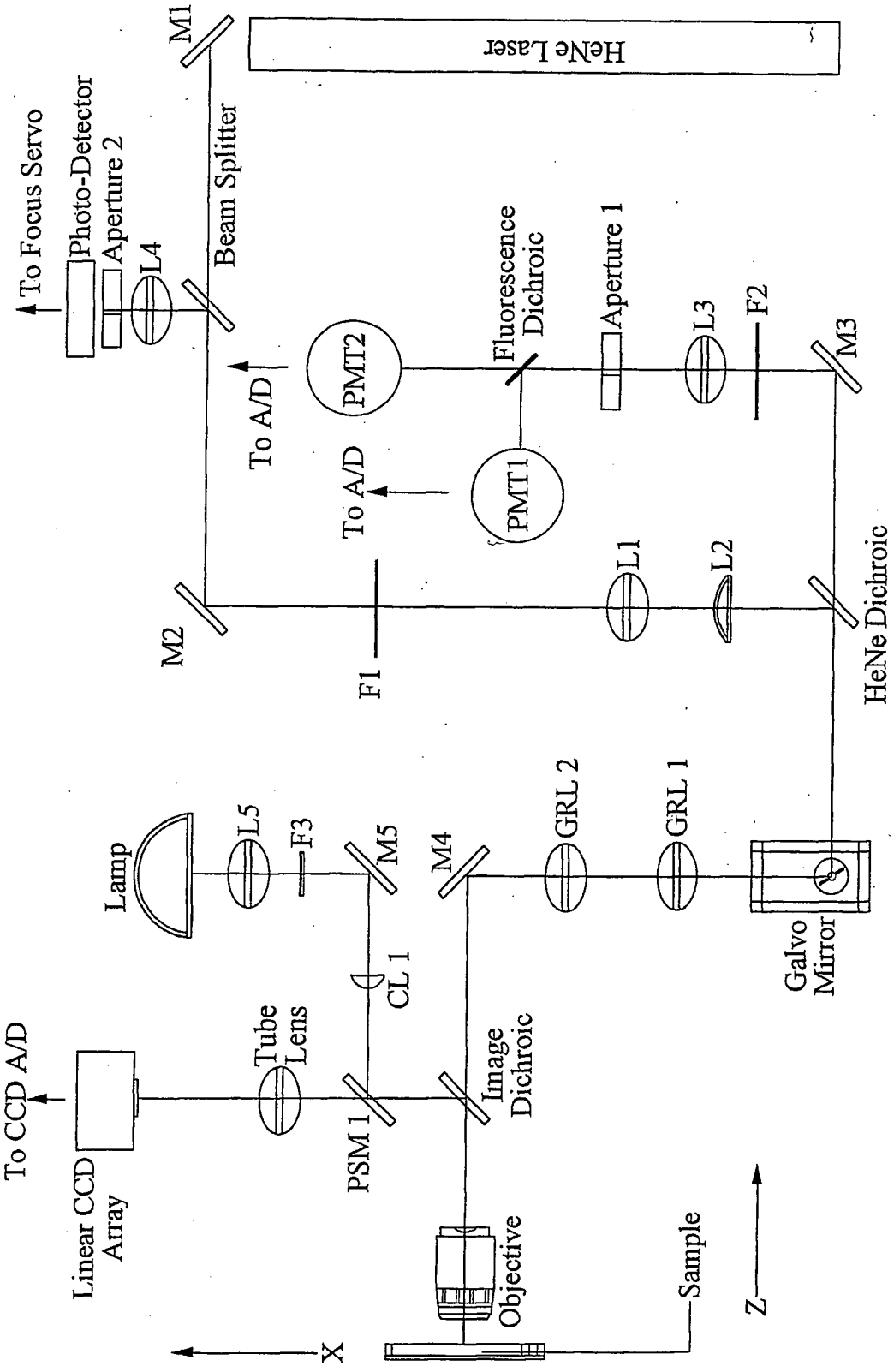

Brightfield/ Fluorescent SM1 Image Pairs
Brightfield Image (BF)
High Pass Filter
Background Assessment
P-P Noise Assessment
Threshold Image
Segmentation
Test Size
Discard
Thinning
Test Length
Discard
Line Fit
Test Fit
Discard
Correlate BF image with user-defined profiles
Fluorescent Image (FL)
Use segmented blobs to determine pixel area over which to assess fluorescent intensity
Store Nanobar Profile/ Fluorescent Information
Loop to next SM1 image pair and append data

Fig. 7


HeNe Laser
M1
Beam Splitter
To Focus Servo
Photo-Detector
Aperture 2
L4
To A/D
PMT2
Fluorescence Dichroic
Aperture 1
L3
F2
M3
To A/D
PMT1
F1
M2
L1
L2
HeNe Dichroic
Lamp
L5
F3
M5
M4
GRL 2
GRL 1
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Tube Lens
Image Dichroic
To CCD A/D
Linear CCD Array
PSM 1
Objective
Sample
X
Z

Fig. 8

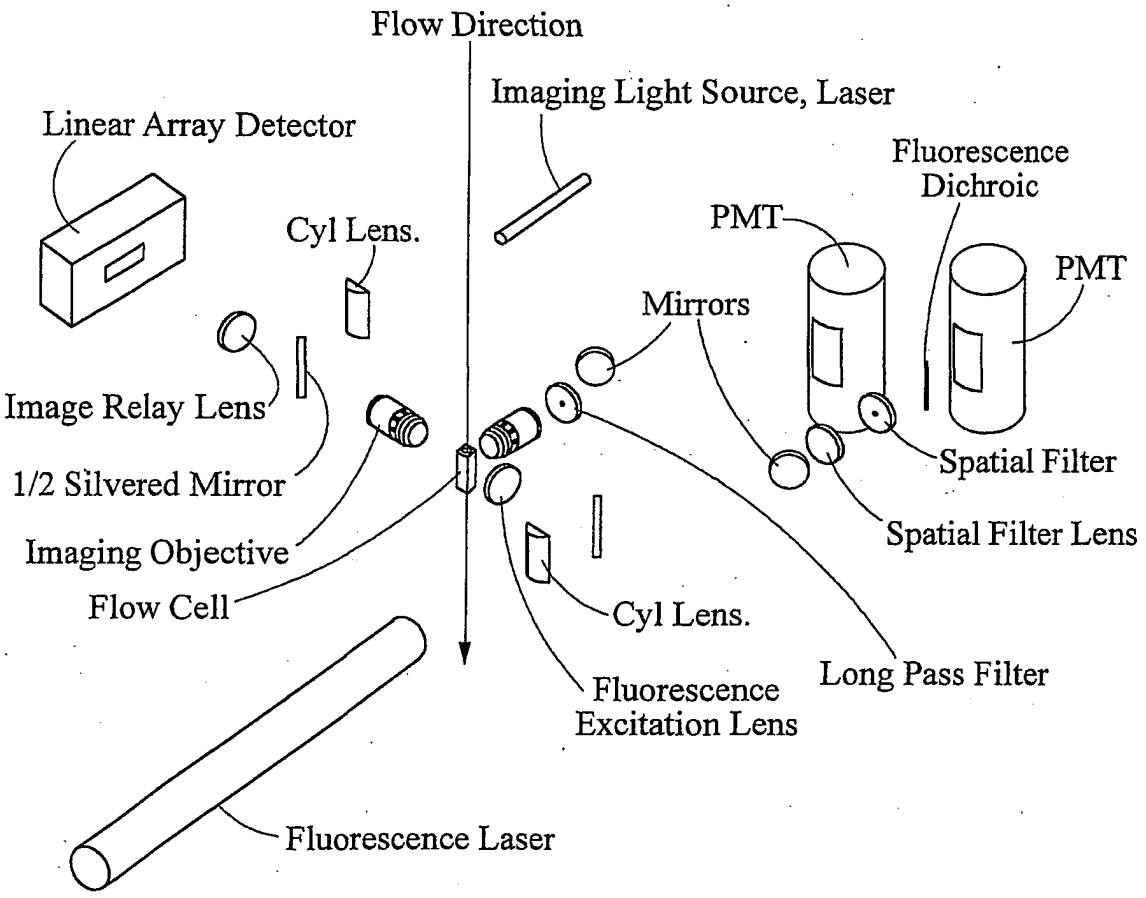
Flow Direction
Imaging Light Source, Laser
Linear Array Detector
Fluorescence Dichroic
PMT
Cyl Lens.
PMT
Mirrors
Image Relay Lens
1/2 Silvered Mirror
Spatial Filter
Imaging Objective
Spatial Filter Lens
Flow Cell
Cyl Lens.
Long Pass Filter
Fluorescence Excitation Lens
Fluorescence Laser

Fig. 9

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27121

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : G06K 9/00
US CL : 382/100, 128

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 382/133, 100, 128, 173, 181, 224; 250/330; 356/301; 372/23; 600/476

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, NPL, IEEE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 5,303,710 A (BASHKANSKY et al.) 19 April 1994, column 5, lines 10-65. | 1-22 |
| Y | US 4,679,939 A (CURRY et al.) 14 July 1987, column 1, lines 35-40; column 6, lines 25-43); column 11, lines 25-65. | 1-22 |
| Y | US 5,091,188 A (HAYNES) 25 February 1992, column 1, lines 6-40; column 19, lines 30-50; column 29, lines 13-35. | 1-22 |
| A | US 3,897,284 A (LIVESAY) 29 July 1975, column 3, lines 40-65. | 1-22 |
| A | US 5,508,164 A (KAUSCH et al.) 16 April 1996, figure 17B, elements 120, 122, 124; column 16, lines 25-60. | 1-22 |
| A | US 5,512,131 A (KUMAR et al.) 30 April 1996, figures 3(a)-(f); column 6, lines 27-60; column 19, lines 1-15. | 1-22 |
| A | US 4,855,930 A (CHAO et al.) 08 August 1989, column 9, lines 40-60. | 7-12 |
| A | US 5,846,517 A (UNGER) 08 December 1998, column 6, lines 25-40; column 10, lines 5-35; column 49, lines 5-35. | 1-22 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 28 November 2000 (28.11.2000) | 04 JAN 2001 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703)305-3230 | Authorized officer<br>Jay Patel<br>Telephone No. (703) 308-7728 |

Form PCT/ISA/210 (second sheet) (July 1998)